

UC Berkeley

UC Berkeley Previously Published Works

Title

Sequence elements controlling expression of Barley stripe mosaic virus subgenomic RNAs in vivo.

Permalink

<https://escholarship.org/uc/item/3p33h9x2>

Journal

Virology, 313(1)

ISSN

1096-0341

Authors

Johnson, Jennifer A
Bragg, Jennifer N
Lawrence, Diane M
et al.

Publication Date

2003-08-01

DOI

10.1016/s0042-6822(03)00285-x

Peer reviewed



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Sequence elements controlling expression of *Barley stripe mosaic virus* subgenomic RNAs in vivo

Jennifer A. Johnson, Jennifer N. Bragg, Diane M. Lawrence, and Andrew O. Jackson*

Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA

Received 17 December 2002; returned to author for revision 17 January 2003; accepted 19 March 2003

Abstract

Barley stripe mosaic virus (BSMV) contains three positive-sense, single-stranded genomic RNAs, designated α , β , and γ , that encode seven major proteins and one minor translational readthrough protein. Three proteins ($\alpha\alpha$, $\beta\alpha$, and $\gamma\alpha$) are translated directly from the genomic RNAs and the remaining proteins encoded on RNA β and RNA γ are expressed via three subgenomic messenger RNAs (sgRNAs). sgRNA β 1 directs synthesis of the triple gene block 1 (TGB1) protein. The TGB2 protein, the TGB2' minor translational readthrough protein, and the TGB3 protein are expressed from sgRNA β 2, which is present in considerably lower abundance than sgRNA β 1. A third sgRNA, sgRNA γ , is required for expression of the $\gamma\beta$ protein. We have used deletion analyses and site-specific mutations to define the boundaries of promoter regions that are critical for expression of the BSMV sgRNAs in infected protoplasts. The results reveal that the sgRNA β 1 promoter encompasses positions -29 to -2 relative to its transcription start site and is adjacent to a *cis*-acting element required for RNA β replication that maps from -107 to -74 relative to the sgRNA β 1 start site. The core sgRNA β 2 promoter includes residues -32 to -17 relative to the sgRNA β 2 transcriptional start site, although maximal activity requires an upstream hexanucleotide sequence residing from positions -64 to -59 . The sgRNA γ promoter maps from -21 to $+2$ relative to its transcription start site and therefore partially overlaps the $\gamma\alpha$ gene. The sgRNA β 1, β 2, and γ promoters also differ substantially in sequence, but have similarities to the putative homologous promoters of other *Hordeiviruses*. These differences are postulated to affect competition for the viral polymerase, coordination of the temporal expression and abundance of the TGB proteins, and constitutive expression of the $\gamma\beta$ protein.

© 2003 Elsevier Science (USA). All rights reserved.

Introduction

The synthesis of subgenomic messenger RNAs (sgRNAs) is a common strategy employed by positive-sense RNA viruses to mediate expression and regulation of 3' proximal open reading frames (ORFs) on multicistronic genomic RNAs. Three general mechanisms have been proposed for sgRNA synthesis (White, 2002). The most commonly accepted mechanism, internal initiation from negative-sense RNA templates (Miller et al., 1985), appears to be operating during sgRNA synthesis by the *Bromoviruses* (Seigel et al., 1997, 1998), *Alfamoviruses* (Van Der Kuyl et al., 1990), *Turnip crinkle virus* (TCV) (Wang and Simon,

1997), and several other viruses (Miller and Koev, 2000). In contrast, a discontinuous mechanism of transcription that produces sgRNAs with 5' and 3' sequences identical to the genomic RNAs has been reported for the large *Coronaviruses* (Sawicki and Sawicki, 1998) and *Arterioviruses* (Pasternak et al., 2001). These sgRNAs arise from minus-strand subgenomic templates produced via polymerase jumping during transcription from genomic RNA templates. A third mechanism, premature termination, has been reported for *Flock house virus* (FHV) (Zhong and Ruekert, 1993; Lindenbach et al., 2000), *Red clover necrotic mosaic virus* (RCNMV) (Sit et al., 1998), and *Tomato bushy stunt virus* (TBSV) (reviewed by White, 2002). In these cases, truncated negative-sense RNAs that had been generated by premature termination at regions of secondary structure of genomic RNA are postulated to serve as templates for the positive-sense sgRNAs. In the case of FHV and TBSV, *cis*-acting sequences within the genomic RNA are postu-

* Corresponding author. Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720. Fax: +1-510-642-9017.

E-mail address: andyoj@uclink4.berkeley.edu (A.O. Jackson).

lated to mediate long distance interactions that contribute to premature polymerase termination during transcription of the negative-sense templates (White, 2002), whereas in the case of RCNMV, termination during transcription from the RNA 1 template requires base pairing of *trans*-activator sequences residing on RNA 2 with *trans*-activator binding sequences on RNA 1 (Sit et al., 1998). Other less well-defined examples of premature termination appear to occur during synthesis of two classes of 5' coterminal sgRNAs appearing in *Citrus tristiza virus* (CTV) infected plants (Che et al., 2001). Although the possible functions of the CTV sgRNAs have not been resolved, the synthesis of the two species appears to be regulated because they exhibit some differences in the timing of their appearance and they are the earliest and the most abundant sgRNAs synthesized.

The core sgRNA promoters have been mapped and characterized in a variety of plant RNA viruses that synthesize either single or multiple sgRNAs (Miller and Koev, 2000). These promoters range in size from less than 30 nucleotides (nt) (Johnston and Rochon, 1995; Wang et al., 1999) to nearly 150 nt (Van Der Vossen et al., 1995; Koev and Miller, 2000), and they normally reside upstream of, or encompass only a few nucleotides downstream of, the transcription initiation site. However, a few promoters include substantial regions downstream of the transcription initiation site (Balmori et al., 1993; Koev and Miller, 2000). For example, the *Beet necrotic yellow vein virus* (BNYVV) RNA3 sgRNA promoter occupies more than 100 nt downstream of the transcription start site (Balmori et al., 1993) and the *Barley yellow dwarf virus* (BYDV) sgRNA2 promoter resides entirely within the sgRNA. In addition to this complexity and variation among RNA viruses, long distance interactions have also been noted in several viruses (Miller and Koev, 2000). In the case of *Tobacco mosaic virus* (TMV), the 3' untranslated region (UTR) contains three pseudoknot structures whose ectopic placement appears to redistribute polymerase activity to the closest upstream sgRNA promoter (Shivprasad et al., 1999). In contrast, optimal activity of the TBSV sgRNA2 promoter requires long distance *cis* interactions with complementary upstream sequences separated by more than 1000 nt (Zhang et al., 1999; Choi and White, 2002).

Promoter elements within viruses expressing more than one sgRNA can also vary considerably in their core sequences, and in their sizes and positioning relative to the transcription start sites of the sgRNAs. In one well-studied case, the BYDV sgRNA1 promoter has been mapped from –75 to +21 relative to its transcription initiation site, while the sgRNA2 and sgRNA3 promoters map between positions +1 and +143, and –6 and +38, respectively (Koev and Miller, 2000). Thus, the BYDV sgRNA1 and sgRNA3 promoters occupy sequences that overlap the transcription initiation site, whereas the sgRNA2 promoter resides entirely within the sgRNA transcript. Aside from a conserved hexanucleotide shared between the sgRNA1 and sgRNA2 promoters, little or no similarity exists between the three pro-

moters. Several lines of evidence suggest that both primary sequence and secondary structure, including two stem loop structures, function during the regulation of BYDV sgRNA1 synthesis.

In contrast to the well-defined viruses described above, only rudimentary information is available to define sequences affecting expression of the sgRNAs of a number of other viruses, including *Barley stripe mosaic virus* (BSMV). BSMV is a *Hordeivirus* whose genome is divided into three positive-sense, single-stranded RNAs, designated α , β , and γ (Fig. 1). The α and γ RNAs are required for replication, while RNA β is essential for cell-to-cell movement. The replicase proteins $\alpha\alpha$ and $\gamma\alpha$ are translated directly from their respective genomic RNAs (Petty et al., 1990). In addition, RNA γ also encodes a small cysteine-rich protein, designated γb , that is translated from sgRNA γ and is dispensable for BSMV replication. The coat protein, βa , is translated directly from RNA β and, following an intergenic region, the overlapping viral movement genes are arranged in a "triple gene block" (TGB). The three major TGB proteins (TGB1, 2, and 3) and one minor protein, TGB2', are translated from two sgRNAs, designated sgRNA β 1 and sgRNA β 2. The TGB1 protein is expressed from sgRNA β 1, while the other overlapping proteins TGB2, TGB2', and TGB3 are translated from sgRNA β 2 (Zhou and Jackson, 1996b). The TGB2' protein is a translational readthrough product of the TGB2 ORF, and the TGB3 protein is translated by leaky scanning of the TGB2 start codon (Zhou and Jackson, 1996b). The transcription initiation sites of the three BSMV sgRNAs have been mapped and their relative abundance has been determined (Gustafson et al., 1987; Zhou and Jackson, 1996b). Northern blot analyses of nucleic acids extracted from BSMV-infected protoplasts have demonstrated that sgRNA γ and sgRNA β 1 accumulate to high levels, whereas sgRNA β 2 is present in much lower abundance (Zhou and Jackson, 1996a, 1996b). Limited evidence from protoplasts also indicates that sgRNA β 1 and β 2 expression is regulated temporally and that sgRNA γ is expressed constitutively (Zhou and Jackson, 1996b).

To begin to determine how expression of the three BSMV sgRNAs may be mediated, we have delineated the boundaries of the promoters based on the expression of sgRNAs in infected protoplasts. In addition, we have mapped a *cis*-acting element adjacent to the sgRNA β 1 promoter that is required for RNA β replication (Zhou and Jackson, 1996b). The three BSMV sgRNA promoters have also been repositioned into β and γ RNAs that either contain or lack the native promoters. The results indicate that the analogous BSMV sgRNA promoters have some sequence similarity to predicted promoter regions of other *Hordeiviruses*. Nevertheless, the three sgRNA promoters do not share extensive sequence similarity, nor are recognizable common structural elements evident. Although the BSMV core promoter sequences failed to function when inserted ectopically into RNA β and RNA γ , core promoters introduced with additional flanking sequences were active,

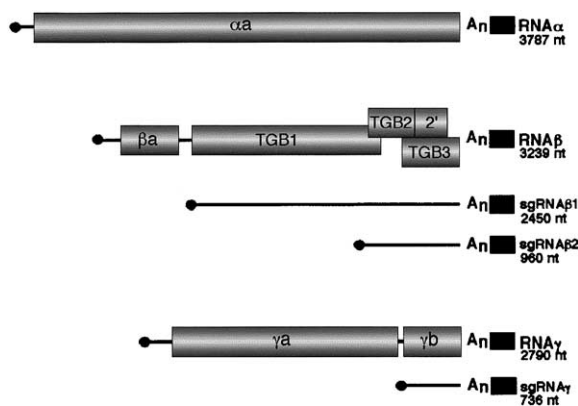


Fig. 1. Illustration of the BSMV genomic and subgenomic RNAs. RNA α serves as the mRNA for the α A protein, which contains the capping and helicase subunits of the RNA-dependent RNA polymerase (RdRp). RNA β encodes the β A protein (coat protein) and the “triple gene block” composed of the TGB1, TGB2, TGB2', and TGB3 proteins. The β A protein is translated from the genomic RNA; the TGB1 protein is expressed from sgRNA β 1 and the TGB2, TGB2', and TGB3 proteins are translated from sgRNA β 2. The genomic RNA γ serves as a messenger for translation of the γ A polymerase subunit of the RdRp and encodes the γ B protein, which is expressed from sgRNA γ . All genomic and subgenomic RNAs are capped (black circle) at the 5' terminus, contain an internal poly (A) tail (A_n), and possess a conserved tRNA-like structure (black rectangle) at the 3' terminus.

but this activity was dependent on the context into which the sequences were inserted.

Results

Mapping sequences required for expression of sgRNA γ

In a previous study, we had determined that the sgRNA γ transcription start site begins at nt 2054 on RNA γ (Gustafson et al., 1987). This site resides within the 42-nt intergenic region between the γ A and γ B ORFs (Fig. 1). To analyze the sgRNA γ promoter, BY-2 protoplasts were transfected with in vitro transcripts, and the RNAs synthesized during infection were evaluated by Northern blot analyses. Because the γ A stop codon is located 18 nt upstream of the sgRNA γ transcriptional start site, we predicted that the sgRNA γ promoter would overlap the γ A ORF. The γ A protein encodes the viral polymerase and hence is essential for viral replication. Therefore, engineering deletions to map the sgRNA γ promoter could have interfered with the γ A ORF and might have affected replication. To circumvent this problem, protoplasts were transfected with two RNA γ derivatives. One RNA, γ KpnI/HpaI, provided a source of the γ A replicase protein and also contained a 350-nt deletion (from positions 2112 to 2461 on RNA γ) to remove the majority of the γ B ORF, and a second derivative designed to assess promoter activity contained deletions engineered within the putative sgRNA γ promoter (Fig. 2A). The deletion present in γ KpnI/HpaI RNA al-

lowed us to distinguish between the two RNA γ derivatives based on their sizes. Protoplasts were transfected with RNA α , RNA γ , and RNA γ KpnI/HpaI, and nucleic acid was extracted from protoplasts at 20 h posttransfection. Each of the three RNAs replicated to wild-type (wt) levels in protoplasts and synthesized the predicted sgRNAs (data not shown). Therefore, in subsequent mapping experiments,

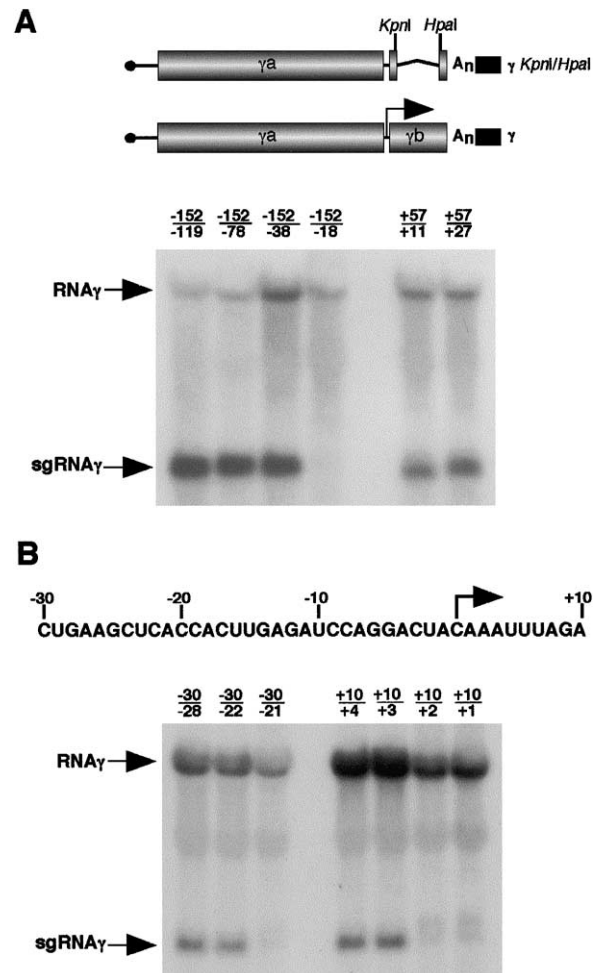


Fig. 2. Identification of the boundaries of the sgRNA γ promoter. (A) The schematic illustration shows the two γ RNAs used in conjunction with RNA α to transfect protoplasts. The γ KpnI/HpaI RNA provided a source of the γ A protein, while the second RNA γ was used to construct deletions within the region surrounding the sgRNA γ transcription start site (note arrow). Tobacco BY-2 protoplasts were cotransfected with the α and γ KpnI/HpaI RNAs plus RNA γ derivatives with deletions originating at either -152 or $+57$ relative to the sgRNA γ transcription start site. The deletions are shown above the blot. Total nucleic acid was extracted 20 h posttransfection, separated on 1% agarose gels, and transferred to nylon membranes. Northern blot analyses were conducted with a γ -specific riboprobe designed to detect the presence of the genomic RNA γ and sgRNA γ . The probe was derived from the KpnI/HindIII (2111 to 2444 nt) fragment of RNA γ and hence does not hybridize to the genomic or sgRNAs generated from the γ KpnI/HpaI RNA. (B) Analysis of RNA γ derivatives containing small deletions in the region spanning -30 to $+10$ relative to the sgRNA γ transcription start site. The sequence shows the intergenic region in the negative sense, with the arrow representing the sgRNA γ transcription start site.

protoplasts were transfected with RNA α , RNA γ *KpnI/HpaI*, and an RNA γ derivative that contained a deletion within the putative sgRNA γ promoter.

To identify starting points for analysis of the sgRNA γ promoter region, two RNA γ derivatives were constructed. One contained a large-scale deletion in the γ a ORF from nt 593 to 1899 and the other had a smaller deletion from positions 2111 to 2262 in the γ b ORF. When these RNAs were transfected individually into protoplasts along with RNA α and RNA γ *KpnI/HpaI*, both of the deletion derivatives were able to replicate and synthesize sgRNA γ at levels comparable to wt RNA γ (data not shown). These results indicate that the deleted regions are not required for sgRNA γ synthesis and that they do not contain regulatory elements that affect replication. Therefore, a more refined analysis of the sgRNA γ promoter was initiated by evaluation of deletions beginning at position -152 (position 1902 on RNA γ) and extending to positions -119 , -78 , -38 , and -18 upstream of the transcription start site. Two additional deletions beginning at position $+57$ and extending to $+27$ and $+11$ were also generated. To specifically visualize the replication of these RNA γ derivatives and the presence of sgRNA γ , a ^{32}P -labeled riboprobe was used that is complementary to the *KpnI/HpaI* region that had been eliminated in the γ *KpnI/HpaI* RNA used to mediate replication of the test RNAs. This probe thus recognizes only the RNA γ and sgRNA γ derivatives designed to assess promoter activity. As shown in Fig. 2A, Northern blot analysis revealed that each of the RNA γ test derivatives were able to replicate *in trans* and that the γ RNAs containing the deletions $-152/-119$, $-152/-78$, $-152/-38$, $+57/+27$, and $+57/+11$ were able to synthesize sgRNA γ . However, when the region between -152 and -18 was deleted, sgRNA synthesis was not evident. Therefore, these results indicated that the sgRNA γ promoter resides between positions -38 and $+11$ relative to the sgRNA γ transcriptional start site.

To analyze the sgRNA γ promoter more precisely, smaller deletions were generated in the region between positions -30 and $+10$ (Fig. 2B). The γ RNAs containing the deletions $-30/-28$, $-30/-22$, $-30/-21$, $+10/+4$, $+10/+3$, $+10/+2$, and $+10/+1$ were transfected into protoplasts along with the α and γ *KpnI/HpaI* RNAs. Northern blot analysis demonstrated that the RNAs containing deletions $-30/-28$ and $-30/-22$ replicated and were capable of synthesizing sgRNA γ , but that removal of the residue at position -21 eliminated sgRNA γ synthesis (Fig. 2B). Our interpretation of these results is that the C at position -22 is dispensable for sgRNA γ synthesis, but that the A at position -21 is required for promoter activity. The RNAs used for deletion analysis starting at position $+10$ were all able to replicate, but only the $+10$ to $+4$ or $+3$ deletions were able to synthesize sgRNA γ (Fig. 2B). When the next residue at $+2$ was deleted, sgRNA γ was not evident, and hence, the A at position $+2$ is required for sgRNA γ synthesis, whereas the A at $+3$ is dispensable. Therefore, these results indicate that the sgRNA γ promoter boundaries map

to positions -21 and $+2$ relative to the sgRNA γ transcription start site. The sgRNA γ promoter thus overlaps the sgRNA γ transcription initiation site and the last two codons of the γ a polymerase protein.

Defining the sgRNA β 1 promoter and the RNA β cis-acting element boundaries

RNA β is dispensable for replication and therefore, substantial deletions could be engineered into the RNA to facilitate analysis of the sgRNA β 1 promoter. The sgRNA β 1 transcription start site had previously been mapped to nt 789 in the 118-nt intergenic region between the β a and TGB1 ORFs (Zhou and Jackson, 1996b). In addition, we had identified a *cis*-acting element required for RNA β replication within this intergenic region (Zhou and Jackson, 1996a). Therefore, careful consideration of the *cis*-acting element had to be taken into account because of the possibility that the sgRNA β 1 promoter boundaries might reside within this region. Hence, we devised a strategy to identify the *cis* element and to map the sgRNA β 1 promoter by transfecting BY-2 protoplasts with the α and γ RNAs and an RNA β mutant to evaluate promoter activity. Northern blot analysis with a β -specific probe was then used to assess replication and sgRNA β 1 synthesis.

Identification of the sgRNA β 1 promoter initially was complicated because the 18S rRNA masked sgRNA β 1 in Northern blots (data not shown). To circumvent this problem, we constructed mutations in a cDNA clone that contained a 1427-nt deletion (β S/B) which removed the majority of the TGB ORFs. This deletion did not affect RNA β replication, in agreement with our previous results (Zhou and Jackson, 1996a) and also clearly resolved sgRNA β 1 and the rRNAs to permit detection of sgRNA β 1 (data not shown). It is important to note that the β S/B RNA deletion also eliminated the sgRNA β 2 promoter to mitigate possible competition effects that might have affected synthesis of sgRNA β 1. To define the starting points for mapping the sgRNA β 1 promoter, we used two RNA β derivatives. One of these, $\beta\Delta 1.6$, contained a deletion from position 295 to 633, and the other, $\beta\Delta 2.0$, eliminated nts 802 to 1375 on the genomic RNA sequence. Northern blot analysis of RNA isolated from protoplasts transfected with the α and γ RNAs and the $\beta\Delta 1.6$ or $\beta\Delta 2.0$ RNAs indicated that the deletions had no discernable effect on sgRNA β 1 synthesis (data not shown). Therefore, the *Bst*BI site at -156 (RNA β nt 633) and the *Nco*I site at $+14$ (nt 802) relative to the sgRNA β 1 transcription start site were used as starting points for analysis.

Initially, deletions were generated from -156 to -107 , -74 and -34 relative to the sgRNA β 1 transcription initiation site. As shown in Fig. 3A, the $-156/-107$ deletion mutant was able to replicate and to synthesize sgRNA β 1, but the $-156/-74$ and $-156/-34$ deletions reduced replication to barely detectable levels. To confirm the replication of RNAs α and γ , the blots were stripped and reprobed with

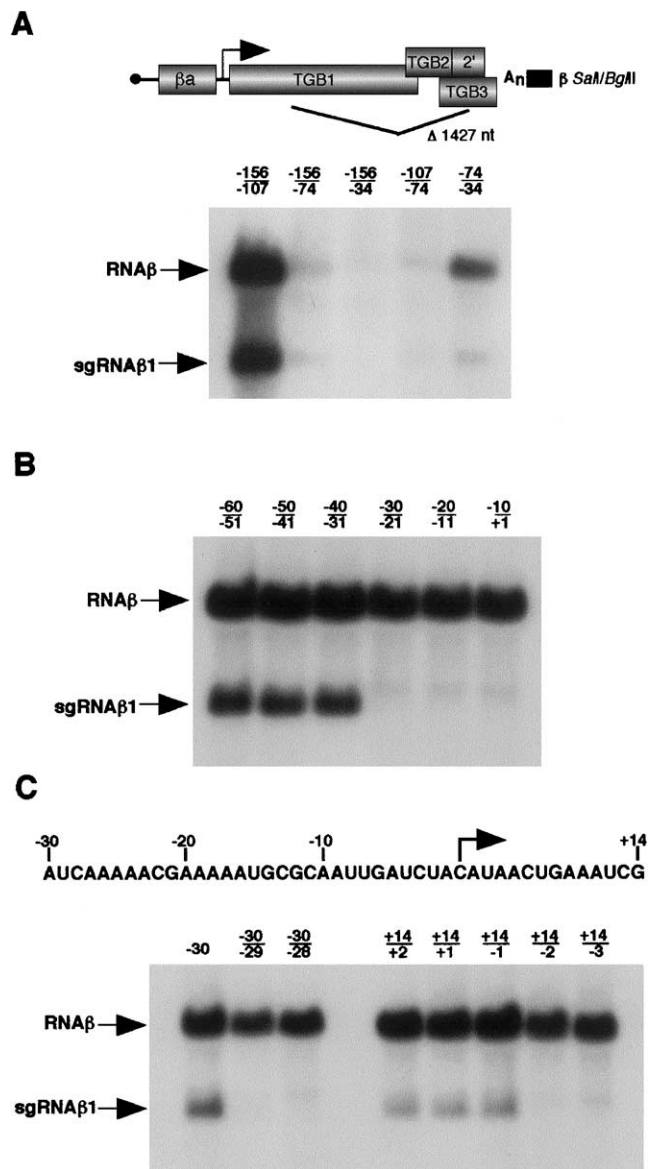


Fig. 3. Delineating the boundaries of the sgRNA β 1 promoter and the RNA β cis-acting element. Protoplasts were transfected with wt α and γ RNAs plus RNA β derivatives containing the 1427 nt S/B (β SalI/BglII) deletion. Total nucleic acid was extracted and blotted as described in Fig. 2, except that the blots were probed with a β -specific riboprobe (β NcoI/SalI). The numbers above the lanes correspond to deletions at positions relative to the sgRNA β 1 transcription start site. (A) Large-scale deletion mapping of the sgRNA β 1 promoter and the RNA β cis-acting element required for replication. (B) Deletion mapping of the sgRNA β 1 promoter using 10-nt deletions at positions corresponding to the transcription start site. (C) Fine-scale mapping of the sgRNA β 1 promoter. The sequence represents the negative-sense orientation of the intergenic region between the βa and TGB1 ORFs which contains the sgRNA β 1 promoter. The arrow represents the sgRNA β 1 transcription start site.

α - and γ -specific probes (data not shown). These results indicated that the -156/-74 and -156/-34 deletions destroyed a cis-element within the intergenic region that is required for replication (Zhou and Jackson, 1996a). To further define the RNA β cis-acting element, two additional

derivatives were constructed that contained deletions spanning -107/-74 and -74/-34. Northern blot analysis using a β -specific probe revealed that replication of -107/-74, similar to that of the -156/-74 and -156/-34 mutants, was undetectable. However, the -74/-34 deletion mutant replicated to low levels and produced small amounts of sgRNA β 1 (Fig. 3A). These results suggest that the cis-acting element required for RNA β replication resides within the 118-nt intergenic region between positions -107 and -74. However, sequences within the -74 to -34 region also contribute to efficient replication of RNA β .

The sgRNA β 1 promoter activity was examined in more detail by engineering 10-nt deletions in β S/B from positions -60 to +1 relative to the sgRNA β 1 transcription start site. Northern blot analyses revealed that the -60/-51, -50/-41, and -40/-31 deletions did not affect genomic RNA replication and abundant amounts of sgRNA β 1 were synthesized. However, when the -30/-21, -20/-11, or -10/+1 regions were deleted, genomic RNA replicated to wt levels but sgRNA β 1 failed to accumulate (Fig. 3B). To further resolve the nature of the active sgRNA promoter element, we engineered smaller deletions from positions -30 to +14. As shown in Fig. 3C, when the A residue at -30 was deleted, sgRNA β 1 was evident, but deletion of the adjacent U at -29 effectively eliminated promoter activity without obvious effects on replication of the genomic RNAs. In contrast, deletions that spanned +14/+2, +14/+1, and +14/-1 retained promoter activity (Fig. 3C). However, when the next residue, a U at -2, was deleted, promoter activity was disrupted. These results suggest that the core promoter sequence required for sgRNA β 1 synthesis resides immediately upstream of the sgRNA β 1 transcription start site between positions -29 and -2. However, an alternative possibility that needs further exploration is that the +14/+1 deletion could have generated an alternative transcription initiation site at the upstream C residue. Similar alternative sites have previously been identified in *in vitro* studies with the *Brome mosaic virus* sgRNA promoter (Stawicki and Kao, 1999).

Delineating the sequences required for sgRNA β 2 expression

The transcription start site of sgRNA β 2 has previously been identified within the 3' end of the TGB1 ORF at position 2279 in RNA β and is 1490 nt downstream of the sgRNA β 1 transcription start site (Zhou and Jackson, 1996b). Since sgRNA β 2 is expressed at very low levels in infected protoplasts (Zhou and Jackson, 1996a, 1996b), we attempted to increase its abundance to improve the ease and reproducibility of promoter analysis. For this purpose, the clone β 1-34/+14 was created by removing nucleotides 755 through 802 (-34/+14 relative to the sgRNA β 1 transcription start site) on RNA β (Fig. 4A). As expected, this 48-nt deletion abrogated sgRNA β 1 synthesis and also resulted in easily detectable levels of sgRNA β 2 without af-

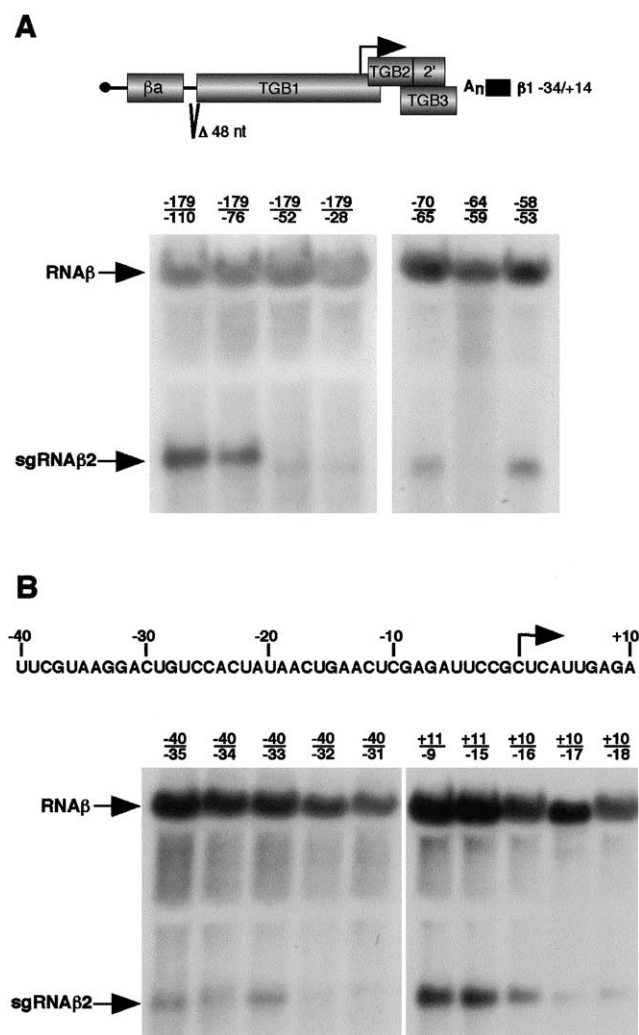


Fig. 4. Defining the boundaries of the sgRNA β 2 promoter. Protoplasts were transfected with the α and γ genomic RNAs and RNA β derivatives containing a 48-nt deletion (β 1–34/+14) to eliminate sgRNA β 1 promoter activity. The deletions are numbered according to the sgRNA β 2 transcription start site, which is illustrated by the arrow. Total nucleic acids were extracted at 20 h posttransfection and processed as described in Fig. 2, except that hybridizations were performed with a β -specific riboprobe (β SspI/BgIII) derived from sequence upstream of the sgRNA2 promoter region. (A) Large-scale deletion mapping of the sgRNA β 2 promoter is shown on the blot to the left. To define the region between –70 and –53 further, 6-nt deletions were constructed and are shown on the blot to the right. (B) Small-scale mapping of the sgRNA β 2 promoter. The sequence represents the negative-sense region of the TGB1 ORF that contains the sgRNA β 2 promoter.

fecting RNA β replication (data not shown). This result implies that competition between the two promoters has a major role in regulating the differential rates of synthesis of the two sgRNAs.

To initially define sequences flanking the sgRNA β 2 promoter, two large deletions were generated in the β 1–34/+14 clone that eliminated RNA β positions 1705 to 2109 and 2287 to 2434. RNAs containing these deletions were individually transfected into protoplasts along with the α and γ RNAs, and nucleic acids were extracted at 20 h

posttransfection. Northern blot analyses using a β -specific probe revealed the presence of sgRNA β 2 in both derivatives, indicating that neither of the deletions affected sgRNA β 2 promoter activity (data not shown). Therefore, we began to focus on the region between –179 (nt 2109) and +9 (nt 2288) relative to the sgRNA β 2 transcription initiation site.

Analysis of four deletions extending from position –179 to positions –110, –76, –52, and –28 relative to the transcription start site revealed that the mutant RNAs were able to replicate in protoplasts, but only mutants containing the deletions –179/–110 or –179/–76 were able to direct synthesis of sgRNA β 2 (Fig. 4A). More detailed analyses performed on the region between –70 and –52 demonstrated that the –70/–65 and –58/–53 mutants transcribed sgRNA β 2, but that promoter activity was destroyed by the –64/–59 deletion (Fig. 4A). These results thus indicate that an upstream element residing between –64 and –59 relative to the sgRNA β 2 transcription start site is required for sgRNA β 2 promoter activity. However, in the presence of this element, deletions between positions –40/–32 and +10/–17 nearly eliminated sgRNA β 2 synthesis (Fig. 4B). In contrast, Northern blot analyses revealed that deletions between –52/–47, –46/–41, –40/–35, –40/–34, and –40/–33 had only minor effects on RNA β replication and sgRNA β 2 synthesis in transfected protoplasts (Fig. 4B and data not shown). Similarly, the deletions +11/–9, +11/–15, and +10/–16 were able to replicate and synthesize sgRNA β 2. These results demonstrate that the sgRNA β 2 promoter maps from the G at –32 to the C at –17 and hence does not encompass the transcriptional start site.

Ectopic expression of the sgRNA promoters

The sgRNA β 1, sgRNA β 2, and sgRNA γ promoter fragments were initially inserted into RNA β to determine whether they could function ectopically and to evaluate their competition with a native sgRNA promoter present on the same RNA. For this purpose, fragments encompassing each of the three sgRNA promoters were PCR-amplified and inserted into RNA β β S/B or β 1–34/+14 at position 1134 (344 nt downstream of the sgRNA β 1 promoter). The β S/B clone provided a background that contained the native sgRNA β 1 promoter (Fig. 5A), while the β 1–34/+14 clone lacked the sgRNA β 1 promoter (Fig. 5B). Two fragments that encompassed the sgRNA β 1 promoter were inserted ectopically into the two RNA β derivatives. The larger fragment β 1a consisted of 266 nt amplified from positions 599 to 864 on the genomic RNA and a smaller 150-nt fragment β 1b was derived from positions 715 to 864. A 295-nt fragment (β 2) overlapping the sgRNA β 2 promoter was derived from positions 2080 to 2374, and a 276-nt fragment (γ) was amplified from positions 1864 to 2139 to encompass the sgRNA γ promoter.

Northern blot analysis of RNA isolated from protoplasts transfected with RNAs α and γ , and RNA β derivatives

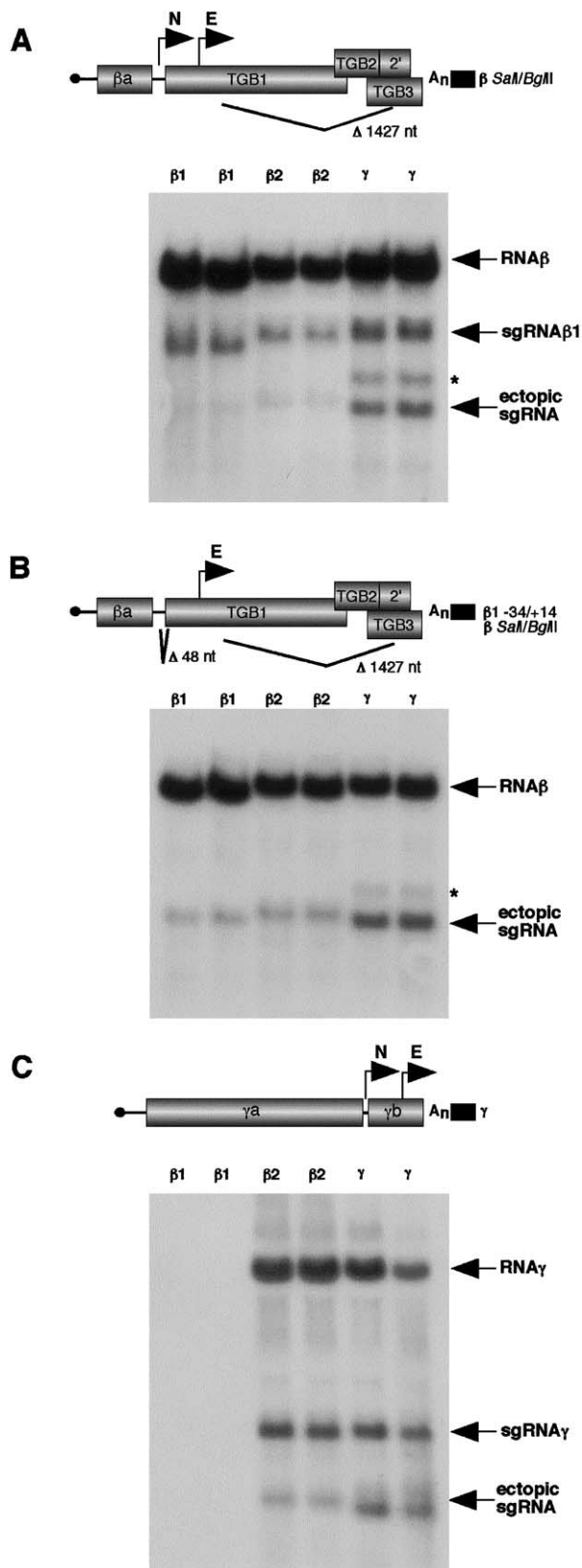


Fig. 5. Ectopic expression of the sgRNA γ , sgRNA β 1, and sgRNA β 2 promoters in RNA β and RNA γ derivatives. The designations on the genomic RNAs are as described in the legend to Fig. 1. N and E represent the native promoters and the ectopic promoter insertion sites, respectively. Protoplast transfections and RNA blots were carried out as described in

containing the native sgRNA β 1 promoter with the smaller sg β 1 (150 nt), sg β 2, or the sg γ fragment insertions at position 1134 revealed that the β RNAs were able to replicate and synthesize sgRNA β 1 from the native promoter (Fig. 5A). However, transcription from the ectopic sgRNA β 1 and sgRNA β 2 promoters could be detected only after protracted exposure of the blots (Fig. 5A, β 1 and β 2 lanes, and data not shown). The RNA β derivative containing the 48-nt deletion in the intergenic region was able to replicate and to express small amounts of the ectopic sgRNAs from the smaller 150-nt sgRNA β 1 fragment and sgRNA β 2 (Fig. 5B, β 1 and β 2 lanes), but both the RNA β derivatives failed to replicate when the larger 266-nt sgRNA β 1 fragment was inserted (data not shown). In marked contrast, the 276-nt ectopic RNA γ fragment was active following insertion into both of the RNA β derivatives and ectopic sgRNAs of the appropriate sizes were easily detectable in both backgrounds (Figs. 5A and B, γ lanes). These results demonstrate that the three BSMV sgRNA promoters can function ectopically in RNA β , but that the extent of their activity varies depending on their flanking sequences and the genomic RNA context into which they are inserted. The results also provide evidence that the ectopic sgRNA γ promoter competes for the BSMV replicase complex more efficiently than an ectopic sgRNA β 1 or sgRNA β 2 promoter when positioned downstream of the wt sgRNA β 1 promoter.

Each of the four promoter fragments (β 1a, β 1b, β 2, and γ) were also inserted into the γ cDNA clone at position 2339, which is located 285 nt downstream of the transcription start site of the native sgRNA γ (Fig. 5C). As seen with RNA β , the presence of the sgRNA β 2 fragment (β 2) and the sgRNA γ promoter derivative (γ) permitted abundant RNA γ replication and synthesis of native sgRNA γ (Fig. 5C, β 2 and γ lanes). Both the sgRNA β 2 and the sgRNA γ promoters were also able to direct synthesis of easily detectable amounts of ectopic sgRNA. Again, transcription from the β 2 promoter was less than half of that from the native sgRNA γ promoter, and the ectopic sgRNA γ promoter also appeared to be substantially more active than the corresponding sgRNA β 2 promoter. However, RNA γ replication

Fig. 2. (A) Activity of the 150 nt sgRNA β 1 (β 1), 294 nt sgRNA β 2 (β 2), and 275 nt sgRNA γ (γ) fragments inserted at nt 1134 of the β S/B (β SalI/BglII) RNA containing the native sgRNA β 1 promoter. Duplicate tubes of BY2 protoplasts were transfected with RNAs α and γ , and either the β S/B+150 nt sg β 1, the β S/B+sg β 2, or the β S/B+sg γ RNA derivatives. RNA blots were probed with a β -specific riboprobe (β c-2785). (B) Expression of the sgRNA β 1, sgRNA β 2, and sgRNA γ promoters in the RNA β S/B derivative (β 1–34/+14), which contains a 48-nt deletion inactivating the native sgRNA β 1 promoter. (C) Activity of the sgRNA β 1, sgRNA β 2, and sgRNA γ promoters inserted into the γ b ORF at nt 2339 of RNA γ . Protoplasts were coinoculated with RNA α and RNA γ containing the promoter fragments and extracted at 20 hpi. RNA blots were probed with a riboprobe that anneals to the conserved 3' end of BSMV RNAs and therefore should detect all genomic and sgRNAs. Asterisks along the side of A and B refer to a consistently observed band of unknown origin that originated from the sgRNA γ promoter insertions.

was nearly eliminated when either the smaller 150-nt sgRNA β 1 promoter (β 1, Fig. 5C) or the larger 266-nt sgRNA β 1 promoter fragments were inserted (data not shown). These results suggest that the ectopic sgRNA β 2 promoter competes less efficiently for the BSMV replicase complex than the wt sgRNA γ promoter. The deleterious effects of the sgRNA β 1 fragment insertions in RNA γ also suggest that sequence, context effects, and perhaps competition for replicase can have drastic effects on replication.

To determine if the three minimal sgRNA promoters were sufficient for ectopic transcription, we inserted each of them into position 1134 of the RNA β β S/B derivative and the 48-nt deletion derivative (β 1–34/+14). Similar insertions were made into RNA γ at position 2339. Ectopic sgRNA expression was not detected following protoplast transfection with any of the derivatives, although the genomic RNAs were able to replicate (data not shown). This result suggests that although the minimal promoter sequences identified by deletion analysis are required for activity in their native context, each of the promoters requires additional flanking sequences to provide optimal promoter activity from the ectopic positions tested in these experiments.

Sequence comparisons of the three BSMV sgRNA promoter regions

The comparisons shown in Fig. 6A indicate that the three BSMV sgRNA promoters share little obvious sequence relatedness. We also conducted a more refined gapped alignment and were still unable to detect major blocks of similarity (data not shown). Additional comparisons of the 238 nt at the 3' common termini of the genomic RNAs and at the diverse 3' ends of the three minus-strand RNAs also indicated that the four replication promoters within the genomic RNAs do not contain substantial regions of similarity to the three BSMV sgRNA promoters (data not shown). However, comparisons of the three sgRNA promoters of BSMV with the putative promoters of *Poa semilatent virus* (PSLV) and *Lychnis ringspot virus* (LRSV) revealed that the analogous *Hordeivirus* sgRNA promoters share a number of blocks of identical sequence (Fig. 6B). Furthermore, the BSMV and PSLV sequences share a higher degree of similarity with each other than either does with LRSV. In the case of the BSMV sgRNA γ promoter, nearly 80% of the PSLV residues are identical (19/24) to the BSMV sequence, whereas a gapped alignment between BSMV and LRSV indicated a considerably lower degree of similarity (14/24 residues) within the aligned region. These results buttress the existing strong evidence for a common origin of the *Hordeiviruses* and support biological evidence, suggesting that BSMV and PSLV are more closely related to each other than to LRSV (Hunter et al., 1989).

To obtain additional information about the relationship of the *Hordeivirus* sgRNA promoters, sequence comparisons were conducted with other TGB-containing viruses.

Initial comparisons using the NCBI BLAST program failed to reveal significant matches with other viral promoter sequences. To obtain more definitive comparisons, we analyzed the 300 nucleotides surrounding the start codons of the TGB proteins of *Beet necrotic yellow vein virus*, *Peanut clump virus* (PCV), *Potato mop top virus* (PMTV), *Potato virus M* (PVM), and *Potato virus X* (PVX). No obvious similarity was detected in comparisons of the sgRNA β 2 promoter regions, and only short blocks of common sequence were noted in the regions surrounding the putative sgRNA β 1 promoters. These common blocks primarily consisted of strings of A residues located at various positions upstream of the translational start site and hence they are unlikely to represent conserved promoter elements. Thus, the results indicate that the sequence conservation noted among the TGB proteins of the *Hordeiviruses* does not extend to the analogous TGB promoter regions of more distantly related viruses.

We also conducted comparisons of the BSMV sgRNA γ promoter with putative promoter regions of other viruses. Interestingly, the PCV p15, which encodes a cysteine-rich protein with some similarity to BSMV γ b, and the BNYVV TGB1 sequences have a high degree of similarity to the BSMV sgRNA γ promoter. In these cases, 16 (PCV) or 14 (BNYVV) residues of 24 are identical to the BSMV sgRNA γ sequence. These regions of similarity include two blocks (ACCACUU and ACUA) within the 20 residues upstream of the transcriptional start site of the BSMV sgRNA γ , and they surprisingly share more than 60% identity (15/24 residues) with a region of the TMV 30 K movement protein promoter (Grzelishvili et al., 2000). Despite these intriguing similarities, the significance, if any, of these common sequences is conjectural and additional analyses are needed to determine their importance in sgRNA synthesis.

Discussion

During the BSMV infection cycle, the three sgRNAs vary in abundance and in their timing of expression (Zhou and Jackson, 1996a). Early in infection, the predominant species are sgRNA γ , which appears to be relatively constant throughout replication, and sgRNA β 1, whose abundance decreases as infection progresses. In contrast, the abundance of sgRNA β 2 is considerably lower than that of sgRNA β 1, but both sgRNAs appear to have similar patterns of temporal expression. These results suggest that the timing and the relative expression levels of the sgRNAs may be important in regulating aspects of the infection cycle (Zhou and Jackson, 1996a). Therefore, understanding the nature of the sgRNA promoters and the requirements for regulated expression of the mRNAs under their control will contribute substantially toward elucidating events required for systemic invasion and disease development in host plants. As a

[illegible]

⁻²⁹
AAUCAAA-----AACGAAAAAUGC**GCAAUUGAU**⁻²**-----CUACAUA** BSMV sgβ1
⁻³²**AAUUUUU-----CAAGA**⁻¹⁷**AAAAAUC****CCUCAUGAA****AA-----CUACA** PSLV sgβ1
UAUAUACAAUUAUACACAAAAAUGAU**CAGACACAGA****UCCUACUUA** LRSV sgβ1

⁻³²**AAGGACU**⁻¹⁷**GUCCACUAUAACUGAACUC****GAGAUUCCG****-----CUCA** BSMV sgβ2
GAAGACU**ACCCACU****-----UACUCACACAGA****UCAUUCG****-----CUGG** PSLV sgβ2
AAGGAU**UACCACUUA****AUUUUAGU****UCUGUUCACG****ACUGA** LRSV sgβ2

⁻²¹**UCUGAAGCU**⁺²**CACCACUUGAGA****UCCAGGACUACAA** BSMV sgγ
CCUGAAGCU**GACCAUUC****CAGA****UCCAGGACU****UCAUA** PSLV sgγ
CCUGAAGCU**AACCACU****CAGGA-----ACUACAUA** LRSV sgγ

[illegible]

Fig. 6. Comparison of the BSMV sgRNA promoter sequences. (A) Sequence of the three BSMV sgRNA promoters in the minus-sense orientation. The numbers above the sequence correspond to nucleotide positions relative to the respective transcription initiation sites. The underlined sequences are required for sgRNA synthesis. (B) Alignment of the sgRNA γ , sgRNA β 1, and sgRNA β 2 promoter regions of the *Hordeiviruses* BSMV, PSLV, and LRSV. Regions with the highest sequence similarity are shown in red. Note that the transcription start sites for the PSLV and LRSV sgRNA promoters have not been defined experimentally, although their initiation sites have been predicted previously (Savenkov et al., 1998). Alignments were performed using the MegAlign program associated with the Lasergene software package. (C) Alignment of the sgRNA β 1, sgRNA β 2, and sgRNA γ promoter regions of BSMV with mapped (TMV and PVX) or putative (BNYVV, PCV, PMTV, and PVM) sgRNA promoter regions of other viruses. Regions identified to have high sequence similarity are shown with residues identical to the BSMV sequence highlighted in red. Alignments were performed using the MegAlign program associated with the Lasergene software package.

step toward such understanding, we have delineated the boundaries of the three BSMV sgRNA promoters.

The sgRNA γ , sgRNA β 1, and sgRNA β 2 promoters map to positions –21 to +2, –29 to –2, and –32 to –17 relative to their transcription start sites, respectively. RNA β also requires an enhancer-like element located between –64 to –59 for production of sgRNA β 2. These three promoters are <50 nt in size and are located in close proximity to their respective transcription initiation sites. The sgRNA γ promoter lies primarily upstream of its transcription initiation site but includes the +1 nt that corresponds to the transcription start site and an additional nucleotide at the +2 position. The BSMV sgRNA β 1 and sgRNA β 2 promoters, which reside upstream of their transcription initiation sites, differ from other plant virus sgRNA promoters previously characterized because the sgRNA β 1 and sgRNA β 2 promoters appear not to overlap their transcription start sites (Miller and Koev, 2000). Independent experiments have demonstrated the importance of the +1 nt in the sgRNA promoters of a number of well-characterized viruses. For example, in vitro studies using the BMV proscript system to elucidate the minimal requirements for replicase recognition of the BMV sgRNA promoter have shown that changing the +1 nt of the promoter from a C to a G decreases sgRNA synthesis by more than 90% (Siegel et al., 1997). The importance of the +1 nt has also been shown in another *Tricornavirus* where changing the C to a U abolished AMV sgRNA promoter activity (Van Der Vossen et al., 1995). Similarly, when the +1 nt of the TMV MP sgRNA promoter was changed from a C to a G, sgRNA accumulation was below the limits of detection (Grdzlishvili et al., 2000). In contrast, transversion of the +1 nucleotides of the BYDV sgRNA2 and sgRNA3 promoters had no effect on sgRNA accumulation, even though both promoters span this region (Koev and Miller, 2000). Although the sgRNA γ promoter overlaps its transcription start site, we have not determined whether nucleotide substitutions at this position affect sgRNA synthesis.

Unlike many RNA viruses, the BSMV sgRNA promoters do not share substantial blocks of sequence identity within their core regions (Fig. 6A). However, the sgRNA γ and sgRNA β 2 promoters do appear to be more closely related to each other than either is to the sgRNA β 1 promoter. This probably is an anomaly due to nucleotide composition within the promoters because the sgRNA β 1 promoter sequence is particularly rich in A and U residues (73%), whereas the sgRNA β 2 and sgRNA γ promoters have a more uniform distribution of bases with an A/U content of just over 50%. In analyses comparing the sgRNA β 1 promoter with either the sgRNA β 2 or the sgRNA γ promoter, we could align no more than two identical residues in a row. However, for the sgRNA β 2 and sgRNA γ promoters, a block of five identical nucleotides (CCACU) can be aligned within the 26 residues upstream of their transcription start sites.

The three BSMV sgRNA promoters align well with the

putative sgRNA promoter regions of the less well-characterized *Hordeiviruses*, PSLV and LRSV, whose sgRNA transcription initiation sites have not been mapped. Comparison of the three BSMV sgRNA promoters with the analogous promoters of the other *Hordeiviruses* reveals over 75% sequence similarity (Fig. 6B and Savenkov et al., 1998). Two blocks of identical residues were identified in the sgRNA β 1 promoter regions (AAAAAU and CUAC). Only one block of conserved sequence was observed for the sgRNA β 2 promoter (CCACU), and this same block is present in BSMV and LRSV sgRNA γ promoters. Among the three *Hordeiviruses*, the sgRNA γ promoter displayed the greatest degree of similarity because it contained three blocks of identical sequence of three nucleotides or longer (GAAGCU, ACCA, and ACU) as well as individual conserved residues. Additional analyses of the less conserved regions failed to indicate preferences for particular purine or pyrimidine residues. These results indicate that the abundance and timing of transcription of the three *Hordeivirus* sgRNAs, β 1, β 2, and γ , may be regulated by promoter sequences that are only distantly related. Alternatively, it is possible that short and/or long distance structural interactions that are not evident in our analyses may have profound roles in promoter timing and activity.

It appears that the sgRNA β 2 promoters of BSMV and PSLV are sufficiently similar for recognition by the BSMV replicase because in vivo studies have revealed that BSMV can support synthesis of PSLV sgRNA β 2 (Solovyev et al., 1999). In these studies, a hybrid BSMV RNA β containing PSLV TGB1, TGB2, and most of the TGB3 sequence was able to replicate and move through the vasculature to uninoculated leaves in plants coinoculated with the α and γ RNAs (Solovyev et al., 1999). However, when a hybrid BSMV RNA β containing the LRSV TGB1, TGB2, and TGB3 regions analogous to the PLSV sequence was coinoculated with the α and γ RNAs, the hybrid virus was unable to infect the same common hosts. These results suggest that the BSMV replicase complex can recognize the related PSLV sgRNA β 2 promoter, but it either cannot recognize the LRSV sgRNA β 2 promoter or the LRSV TGB proteins do not form sufficiently compatible interactions to mediate BSMV movement.

In comparisons of the BSMV sgRNA promoters with the analogous sequences of non-*Hordeivirus* TGB promoters, we were unable to detect a strong correlation between the putative promoter sequences directing sgRNAs encoding specific TGB proteins. For example, no obvious similarity was evident between the sgRNA β 1 promoter and the regions upstream of the PMTV and PCV TGB1 genes, and only limited similarity was detected with the regions upstream of the TGB1 genes of BNYVV, PVM, and PVX. Moreover, an equivalent degree of limited similarity was also observed in the regions upstream of the unrelated BNYVV p14 and the PCV TGB2 genes. Likewise, the sgRNA γ promoter shares small blocks of common sequence with the region upstream of the PCV p15 gene,

which encodes a protein closely related to the γ b protein, but the promoter also has similarity to the regions upstream of the unrelated BNYVV TGB1 and TMV p30 genes. The BSMV, BNYVV, PVM, and PCV replicase proteins belong to the supergroup 3 tobamo lineage of RNA-dependent RNA polymerases (Koonin and Dolja, 1993), so in these cases it is tempting to speculate that the promoter sequences coevolved in response to the polymerase specificity rather than as a block consisting of the promoter and its associated protein. However, the RdRp proteins of BMV and PVX are also classified in the supergroup 3 tobamo lineage, yet their sgRNA promoter sequences share no apparent similarity to the BSMV promoters. Therefore, it is difficult to make a case for promoter evolution based on either promoter protein constraints or polymerase similarity.

We performed computer analyses to search for common secondary structural elements within or flanking the minimal BSMV core promoters, but were unable to identify common elements. This is surprising because in other viruses, sgRNA promoter recognition by replicase complexes often requires primary sequence as well as secondary structural elements. These requirements may facilitate diverse interactions between replicase complexes and promoters with limited sequence similarity. In BYDV, both RNA sequence and secondary structure are essential for promoter activity (Koev et al., 1999). The BYDV sgRNA1 promoter folds into two stem loops downstream of its transcription initiation site, but comparisons with the predicted structures of the sgRNA2 and sgRNA3 promoters reveal that they possess very different sized stem loops downstream of their respective transcription initiation sites (Koev and Miller, 2000). The TMV MP sgRNA promoter also requires a stem loop structure for promoter activity that will tolerate substantial variation in primary sequence without major effects on sgRNA synthesis (Grdzlishvili et al., 2000). In addition, chemical and enzymatic probing of the 1.45-kb sgRNA promoter of TCV has confirmed the presence of a 96-nt hairpin structure, although only a 21-nt hairpin and a 9-nt flanking single-stranded region comprise the minimal promoter (Wang et al., 1999). Thus, our comparisons suggest that BSMV promoters differ from those of a number of other viruses in that conservation of primary sequence rather than conserved structure may have a predominant role in promoter activity.

The available evidence indicates that sequences flanking the core promoter and positioning of the promoter contribute substantially to the transcription of the BSMV sgRNAs. In the case of sgRNA β 2, expression increased substantially upon removal of a 48-nt sequence required for sgRNA β 1 promoter activity. This result suggests that competition for replicase has an important role in mediating the levels of expression of sgRNA β 1 and sgRNA β 2. The ectopic expression experiments support this hypothesis because both the ectopic sgRNA γ and the sgRNA β 2 promoters directed transcription more efficiently in constructs lacking the wt promoters than in constructs with wt promoters. Positioning

also appears to have variable effects on promoter activity. Accumulation of sgRNAs transcribed from promoters located close to the 3' terminus of the genomic-sense RNA was less abundant than those of the wt sgRNA from an upstream promoter. When the sgRNA γ , sgRNA β 1, and sgRNA β 2 promoters were placed downstream of the wt sgRNA β 1 promoter in RNA β , the ectopic sgRNAs were considerably reduced in comparisons with the wt sgRNA β 1 (Fig. 5). However, in RNA γ , the ectopic sgRNA γ promoter strength appeared to be similar to that of the wt sgRNA γ promoter. On the other hand, when the ectopic sgRNA β 2 promoter was placed downstream of the wt sgRNA γ promoter, the ectopic sgRNA accumulated to much lower levels than the wt sgRNA γ (Fig. 5C). In contrast to the larger regions encompassing the BSMV sgRNA promoters, the minimal BSMV promoter sequences were unable to function ectopically. These results suggest that positioning, flanking sequences, and, in the case of sgRNA β 2, an upstream enhancer element may contribute substantially to promoter strength. In particular, the reduction in activity resulting from placement of the BSMV sgRNA promoters near the 3' end of the genomic RNA contrasts with many examples reported from other RNA viruses where more 3' located ectopic promoters are preferentially transcribed (French and Ahlquist, 1988; Boccard and Baulcombe, 1993; Wang and Simon, 1997; Koev and Miller, 2000).

The majority of viruses that contain multiple sgRNA promoters on the same RNA shows a strong correlation between the abundance of their sgRNAs and their proximity to the 3' end of the genomic RNA (Kelly et al., 1994; Wang and Simon, 1997; Grdzlishvili et al., 2000). However, BSMV sgRNA β 1 and sgRNA β 2 promoters appear not to share this correlation because sgRNA β 2, which is proximal to the 3' terminus of RNA β , accumulates to much lower levels than the upstream sgRNA β 1. This pattern of synthesis for TGB sgRNAs has also been demonstrated for *Potato virus X* (Verchot et al., 1998) and is likely the case for the multicomponent *Benyviruses*, *Furoviruses*, *Pomoviruses*, and *Pecluviruses* and the monopartite *Carlaviruses*, *Allexiviruses*, and *Foveaviruses* (van Regenmortel et al., 2000), which contain related TGBs. During replication of BSMV, PVX, and BNYVV, two sgRNAs are generated for translation of the overlapping TGB proteins. The relatively high abundance of sgRNA1 reflects the accumulation of the TGB1 protein during the early stages of infection, whereas the 3' proximal sgRNA β 2 is often difficult to detect (Zhou and Jackson, 1996a, 1996b; Verchot et al., 1998). It is possible that several evolutionary constraints may contribute to the low abundance of sgRNA β 2 and the analogous sgRNA in other TGB-containing viruses. One possibility is that the relative abundance of the proteins encoded by the sgRNAs may be important for the movement complex function. Alternatively, because the sgRNA β 2 promoter resides within the TGB1 ORF, optimal promoter strength may have been constrained by necessities for TGB1 protein function.

Due to the compact nature of plant RNA viruses, se-

Table 1
Synthetic oligonucleotides used in this study

Name	Primer sequence	Description
γ SpeI-119	GCTATTTCTGCA CTAGT TTTCTTTATG	sgRNA γ promoter large-scale mapping
γ SpeI-78	GACTTTAATAAGT ACTAGT TGCTGTTTAATTG	sgRNA γ promoter large-scale mapping
γ SpeI-38	GAAATTTGTGGATAAA CTAGT GAGAAAAGAC	sgRNA γ promoter large-scale mapping
γ SpeI-18	GAAAAGACTTCG ACTAGT GAACTCTAGG	sgRNA γ promoter large-scale mapping
γ SpeI+11	GTTTAAATCT ACTAGT TTTACCTTCGC	sgRNA γ promoter large-scale mapping
β 1KpnI-107	CCAGATGCCGAGG TACCC GTGACCTGCTG	sgRNA β 1 promoter large-scale mapping
β 1KpnI-74	GCGGTAAGGG TACCAT ATGTATC	sgRNA β 1 promoter large-scale mapping
β 1KpnI-34	TATCTATTTTC GGTAC CTTTAGTTTTTG	sgRNA β 1 promoter large-scale mapping
β 2+9SmaI	GGCGAGTAACTCCCGGGGTATTCTGCAA	sgRNA β 2 promoter large-scale mapping
β 2KpnI-110	GGAAAGCTTTAGCAGG TACCAT TTGCGTTTAG	sgRNA β 2 promoter large-scale mapping
β 2KpnI-76	CTTTGAGCAGACAG TACCG AAAGTTAATCATC	sgRNA β 2 promoter large-scale mapping
β 2KpnI-52	GTTAATCATCAGGGCG TACCG AAATTCGTC	sgRNA β 2 promoter large-scale mapping
β 2KpnI-28	ATTCGTCAAGCAT TGGTAC CAGGTGATATTGAC	sgRNA β 2 promoter large-scale mapping
5'sg γ BamHI	GGATCC GGTGCTTGATGCTTTGGATAAG	PCR amplification of sgRNA γ promoter
3'sg γ BamHI	GGATCCCC ACAGTAAGTACTTGTAGTTAAG	PCR amplification of sgRNA γ promoter
5'sg β 1BamHI	GGATCC GAGTGCGAGACTCCAGTG	PCR amplification of sgRNA β 1 promoter
3'sg β 1BamHI	GGATCCC ACCTTTCACTGAATCAG	PCR amplification of sgRNA β 1 promoter
5'sg β 2BamHI	GGATCC GTGCCCTGGCAATTGATGTGCAAG	PCR amplification of sgRNA β 2 promoter
3'sg β 2BamHI	GGATCCCAGACCGATTCCGGCGACAATTGG	PCR amplification of sgRNA β 2 promoter
β 1134BamHI	GCTCAACCGAGT AGGATCC ATGACCTTAAAGG	Engineer BamHI site from nts 1132–1137 in RNA β
γ 2339BamHI	CCAAAAGCATGCG ATCCG TATGATTAC	Engineer BamHI site from nts 2338–2343 in RNA γ
5'sg β 1-74BamHI	GGATCCC ATATGTATCTTATTTATT	PCR amplification of sgRNA β 1 promoter
BSMV3'SpeI	GGGAAGAC CTAGT CATGCAAGC	Change MluI site to SpeI at BSMV RNA γ 3' end

Note. Restriction sites are in bold.

quences residing within the same region of the genome often have evolved to perform multiple functions. This is especially evident in BSMV because the sgRNA β 1 core promoter is proximal to a *cis*-acting element required for RNA β replication, and also for the sgRNA γ and sgRNA β promoters, which overlap regions encoding the γ and TGB1 ORFs, respectively. Since the BSMV sgRNA promoter regions are multifunctional, it is likely that the replicase complex has coevolved with viral *cis*-acting elements to permit recognition of the three different sgRNA promoters and to discriminate between the distinct elements at the 3' ends of the positive- and negative-sense RNAs. These varied interactions may also help explain the temporal regulation of the sgRNA promoters in the same virus. For example, the diverse promoter–replicase interactions within a virus may be facilitated by varied complexes of replicase and host proteins with distinct promoter elements. Such interactions may well mediate the strength and temporal activities of the BSMV sgRNA promoters.

Materials and methods

Recombinant plasmids

The α , β (β 42SpI), and γ (γ 42) cDNA clones used in this study were derived from the BSMV ND18 strain (Petty et al., 1988). To define the starting points for mapping the sgRNA γ promoter, the γ cDNA clone was digested with KpnI and BsmI, the 3' overhangs were removed with T4

DNA polymerase, and the vector was religated to generate the clone $\gamma\Delta$ KpnI/BsmI. The $\gamma\Delta$ E/E cDNA clone constructed by Zhou and Jackson (1996a) was also used. To generate large-scale deletions in the sgRNA γ promoter, SpeI sites were engineered by site-directed mutagenesis (Kunkel, 1985) at positions -119, -78, -38, -18, and +11 relative to the sgRNA γ transcription start site at 2054 nt using the oligonucleotides listed in Table 1. The resulting clones, γ SpeI-119, γ SpeI-78, γ SpeI-38, and γ SpeI-18 were digested with ClaI (-152) and SpeI, while the γ SpeI+11 clone was digested with KpnI (+57) and SpeI. The digested clones were treated with T4 DNA polymerase to generate blunt ends and religated. These clones were designated γ -152/-119, γ -152/-78, γ -152/-38, γ -152/-18, and γ +57/+11. The clone γ +57/+27 was constructed by digesting the γ 42BamHI clone (Petty et al., 1990) with BamHI and KpnI, blunting with T4 DNA polymerase, and religating. Smaller deletions within the sgRNA γ promoter region were engineered by site-directed mutagenesis using the γ cDNA clone to create deletions ranging in size from 2 to 10 nts in the -30 to +10 region surrounding the transcription start site. Throughout the course of this work, deletions or site-directed mutations were sequenced to ensure that the desired changes had been introduced.

To identify the starting points for mapping the sgRNA β 1 promoter, we used the $\beta\Delta$ 1.6 and $\beta\Delta$ 2.0 clones (Petty and Jackson, 1990). Refined mapping of the sgRNA β 1 promoter was performed using a β cDNA clone (β S/B) containing a 1427-nt deletion from SalI to BglII that removes part of the TGB1, TGB2, and TGB3 coding regions. This deletion was

engineered to increase the separation between the sgRNA β 1 and the 18S ribosomal RNA in agarose gels. *Kpn*I sites were introduced into the β S/B cDNA clone at positions –107, –74, and –34 relative to the sgRNA β 1 transcription start site at 789 nt using site-directed mutagenesis and β 1-specific oligonucleotides (Table 1). The resulting clones were digested with *Bst*BI (–156) and *Kpn*I, blunted with T4 DNA polymerase, and religated to generate the β 1–156/–107, β 1–156/–74, and β 1–156/–34 clones. Deletions were engineered in the sgRNA β 1 promoter region of the β S/B clone by site-directed mutagenesis to produce the β 1–60/–51, β 1–50/–41, β 1–40/–31, β 1–30/–21, β 1–20/–11, and β 1–10/+1 clones with a series of sequential deletions from positions –60 to +1. More detailed resolution of the sgRNA β 1 promoter region was obtained by site-directed mutagenesis of the β S/B cDNA clone to generate the clones β 1–30, β 1–30/–29, β 1–30/–28, β 1 +14/+2, β 1, +14/+1, β 1 +14/–1, β 1 +14/–2, and β 1 +14/–3.

To define the *cis*-acting element required for RNA β replication, two *Kpn*I sites were created at –107 and –74, or –74 and –34, relative to the transcription start site using site-directed mutagenesis. These clones were digested with *Kpn*I and religated to produce the clones β 1–107/–74 and β 1–74/–34.

The sgRNA β 2 promoter region was analyzed in a β cDNA background that contained a deletion between –34 and +14 relative to the sgRNA β 1 transcription start site and hence lacked the sgRNA β 1 promoter. Clone β 1–34/+14 was constructed by digesting the β 1–34/*Kpn*I clone with *Kpn*I and *Nco*I, blunting with T4 DNA polymerase, and religating. Since the sgRNA β 2 transcription start site is located at nt 2279, the starting points for mapping the sgRNA β 2 promoter were defined with the clone $\beta\Delta$ *Pst*I/*Eco*RI, which lacks nts 1705 to 2109, and β 2 + 9/*Ssp*I, lacking nts 2290 to 2434. Clone $\beta\Delta$ *Pst*I/*Eco*RI was generated from the β 1–34/+14 clone by digestion with *Pst*I and *Eco*RI, blunting with T4 DNA polymerase, and religation. Clone β 2 + 9/*Ssp*I was engineered by introducing a *Sma*I site at position +9 relative to the sgRNA β 2 transcription start site by site-directed mutagenesis with the oligonucleotide β 2 + 9/*Sma*I (Table 1). The resulting derivative was partially digested with *Ssp*I, digested with *Sma*I, and religated to generate the β 2 + 9/*Ssp*I clone. For large-scale mapping of the sgRNA β 2 promoter, the β 1–34/+14 cDNA clone was used in conjunction with the β 2-specific oligonucleotides listed in Table 1 to engineer unique *Kpn*I sites upstream of the sgRNA β 2 promoter at positions –110, –76, –52, and –28. These clones were subsequently digested with *Eco*RI (–179) and *Kpn*I, blunted with T4 DNA polymerase, and religated to generate the clones β 2–179/–110, β 2–179/–76, β 2–179/–52, and β 2–179/–28.

Six nucleotide deletions from –70 to –35 relative to the sgRNA β 2 transcription start site were generated by site-directed mutagenesis of clone β 1–34/+14 to produce the clones β 2–70/–65, β 2–64/–59, β 2–58/–53,

β 2–52/–47, β 2–46/–41, and β 2–40/–35. More refined mapping of the sgRNA β 2 promoter was facilitated by generating the clones β 2–40/–35, β 2–40/–34, β 2–40/–33, β 2–40/–32, β 2–40/–31, β 2+11/–9, β 2 + 10/–15, β 2 + 10/–16, β 2 + 10/–17, and β 2 + 10/–18 by site-directed mutagenesis of the β 1–34/+14 cDNA clone.

To determine if the three sgRNA promoters can function ectopically, regions surrounding the sgRNA γ (276 nt), sgRNA β 2 (295 nt), or the sgRNA β 1 promoter (256 and 150 nt) were PCR amplified. These reactions were carried out with the oligonucleotides listed in Table 1 to introduce *Bam*HI sites at the 5' and 3' termini. A *Bam*HI site was engineered in the β S/B clone at nt 1132–1137, by site-directed mutagenesis with the β 1134/*Bam*HI oligonucleotide (Table 1). The *Bam*HI site was then introduced into RNA β derivatives that either contained or lacked the sgRNA β 1 promoter (β 1–34/+14). In addition, a *Bam*HI site was engineered into the γ cDNA clone (containing the sgRNA γ promoter) and the γ –30/–21 cDNA clone (lacking the wt sgRNA γ promoter) at nt 2338–2343 with the γ 2239/*Bam*HI oligonucleotide (Table 1). These intermediate plasmids were then digested with *Bam*HI and ligated to each of the amplified subgenomic promoters (sgRNA γ , sgRNA β 1, and sgRNA β 2) to generate clones β 1+sg γ , β 1+sg β 1, β 1+sg β 2, β +sg γ , β +sg β 1, β +sg β 2, γ 1+sg γ , γ 1+sg β 1, γ 1+sg β 2, γ +sg γ , γ +sg β 1 and γ +sg β 2. Since the sgRNA β 1 promoter contains a *Mlu*I site, the γ +sg β 1 and γ +sg β 1 clones were linearized for in vitro transcription reactions at a *Spe*I site introduced at their 3' termini with the BSMV3'*Spe*I oligonucleotide (Table 1).

Protoplast isolation and transfection

Protoplasts from the BY-2 tobacco cell suspension line were prepared and transfected as described previously (Watanabe et al., 1982). To map the sgRNA γ promoter, protoplasts were transfected with in vitro transcripts (Petty et al., 1988) generated from the cDNA clones α and γ *Kpn*I/*Hpa*I (Zhou and Jackson, 1996a), plus one of the modified γ clones described above. For analysis of the sgRNA β 1 and sgRNA β 2 promoters, protoplasts were transfected with in vitro transcripts from the α and γ cDNA clones, plus the appropriate modified β cDNA clone. Prior to use in in vitro transcription reactions, the β cDNA clones were linearized with *Spe*I, and the α and γ cDNA clones were linearized with *Mlu*I, except for the γ 1+sg β 1 and γ +sg β 1 clones which were linearized with *Spe*I.

RNA extraction and analysis

RNA was extracted from BY-2 protoplasts at 20 h post-transfection as described previously (Zhou and Jackson, 1996a). For Northern blot analysis, 5 μ g of total nucleic acid was separated on 1% agarose TBE gels and blotted onto Hybond NX (Amersham Pharmacia Biotech) nylon membranes. Prehybridization, hybridization, and washes

were performed as recommended in the manufacturer's instructions.

RNA β - and RNA γ -specific [32 P]-UTP-labeled riboprobes were generated by in vitro transcription reactions using T7 or T3 RNA polymerase (Zhou and Jackson, 1996a). Portions of the β (2434 to 2785 nt, 802 to 1375 nt) or γ (2111 to 2444 nt) cDNA clones were introduced into pBluescript KS+ (Stratagene) or pGem5Zf+ (Promega, Madison, WI) plasmids and linearized prior to being used in in vitro transcription reactions. An additional RNA β -specific-riboprobe (2785–2985 nt) was generated by linearizing the β c-2785 clone (D.M. Lawrence and A.O. Jackson, unpublished results) with *Bam*HI prior to use in in vitro transcription reactions.

Acknowledgments

We thank Erin Duncan for technical assistance and Teresa Rubio and Sharon Fodor for comments on the manuscript. This research was supported by USDA Competitive Grant 97-35303-4572 and by a grant from Torrey Mesa Research Institute, Syngenta Research and Technology. J.B. was the recipient of a National Science Foundation Graduate Research Fellowship.

References

- Balmori, E., Gilmer, D., Richards, K., Guilley, H., Jonard, G., 1993. Mapping the promoter for subgenomic RNA synthesis on the beet necrotic yellow vein virus RNA 3. *Biochimie* 75, 517–521.
- Boccard, F., Baulcombe, D., 1993. Mutational analysis of *cis*-acting sequences and gene function in RNA3 of cucumber mosaic virus. *Virology* 193, 563–578.
- Che, X., Piestun, D., Mawassi, M., Yang, G., Satyanarayana, T., Gowda, S., Dawson, W.O., Bar-Joseph, M., 2001. 5'-Coterminal subgenomic RNAs in citrus tristeza virus-infected cells. *Virology* 283, 374–381.
- Choi, I.R., White, K.A., 2002. An RNA activator of subgenomic mRNA1 transcription in tomato bushy stunt virus. *J. Biol. Chem.* 277, 3760–3766.
- French, R., Ahlquist, P., 1988. Characterization and engineering of sequences controlling in vivo synthesis of brome mosaic virus subgenomic RNA. *J. Virol.* 62, 2411–2420.
- Grdzeliashvili, V.Z., Chapman, S.N., Dawson, W.O., Lewandowski, D.J., 2000. Mapping of the tobacco mosaic virus movement protein and coat protein subgenomic RNA promoters in vivo. *Virology* 275, 177–192.
- Gustafson, G., Hunter, B., Hanau, R., Armour, S.L., Jackson, A.O., 1987. Nucleotide sequence and genetic organization of barley stripe mosaic virus RNA γ . *Virology* 158, 394–406.
- Hunter, B.G., Smith, J., Fattoh, F., Jackson, A.O., 1989. Relationship of lychnis ringspot virus to barley stripe mosaic virus and poa semilant virus. *Intervirology* 30, 18–26.
- Johnston, J.C., Rochon, D.M., 1995. Deletion analysis of the promoter for the cucumber necrosis virus 0.9-kb subgenomic RNA. *Virology* 214, 100–109.
- Kelly, L., Gerlach, W.L., Waterhouse, P.M., 1994. Characterization of the subgenomic RNAs of an Australian isolate of barley yellow dwarf luteovirus. *Virology* 202, 565–573.
- Koev, G., Miller, W.A., 2000. A positive-strand RNA virus with three very different subgenomic RNA promoters. *J. Virol.* 74, 5988–5996.
- Koev, G., Mohan, B.R., Miller, W.A., 1999. Primary and secondary structural elements required for synthesis of barley yellow dwarf virus subgenomic RNA1. *J. Virol.* 73, 2876–2885.
- Koonin, E.V., Dolja, V.V., 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28, 375–430.
- Kunkel, T.A., 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82, 488–492.
- Lindenbach, B.D., Sgro, J.Y., Ahlquist, P., 2002. Long-distance base pairing in flock house virus RNA1 regulates subgenomic RNA3 synthesis and RNA2 replication. *J. Virol.* 76, 3905–3919.
- Miller, W.A., Dreher, T.W., Hall, T.C., 1985. Synthesis of brome mosaic virus subgenomic RNA in vitro by internal initiation on minus-sense genomic RNA. *Nature* 313, 68–70.
- Miller, W.A., Koev, G., 2000. Synthesis of subgenomic RNAs by positive-strand RNA viruses. *Virology* 273, 1–8.
- Pasternak, A.O., van den Born, E., Spaan, W.J., Snijder, E.J., 2001. Sequence requirements for RNA strand transfer during nidovirus discontinuous subgenomic RNA Synthesis. *EMBO J.* 20, 7220–7228.
- Petty, I.T.D., French, R., Jones, R.W., Jackson, A.O., 1990. Identification of barley stripe mosaic virus genes involved in viral RNA replication and movement. *EMBO J.* 9, 3453–3457.
- Petty, I.T.D., Hunter, B.G., Jackson, A.O., 1988. A novel strategy for one-step cloning of full-length cDNA and its application to the genome of barley stripe mosaic virus. *Gene* 74, 423–432.
- Petty, I.T.D., Jackson, A.O., 1990. Mutational analysis of barley stripe mosaic virus RNA β . *Virology* 179, 712–718.
- Savenkov, E.I., Solovyeve, A.G., Morozov, S.Y., 1998. Genome sequences of poa semilant and lychnis ringspot hordeiviruses. *Arch. Virol.* 143, 1379–1393.
- Sawicki, S.G., Sawicki, D.L., 1998. A new model for coronavirus transcription. *Adv. Exp. Med. Biol.* 44, 215–219.
- Shivprasad, S., Pogue, G.P., Kewandowski, D.J., Hidalgo, J., Donson, J., Grill, L.K., Dawson, W.O., 1999. Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology* 255, 312–323.
- Siegel, R.W., Adkins, S., Kao, C.C., 1997. Sequence-specific recognition of a subgenomic RNA promoter by a viral RNA polymerase. *Proc. Natl. Acad. Sci. USA* 94, 11238–11243.
- Siegel, R.W., Bellon, L., Beigelman, L., Kao, C.C., 1998. Moieties in an RNA promoter specifically recognized by a viral RNA-dependent RNA polymerase. *Proc. Natl. Acad. Sci. USA* 95, 11613–11618.
- Sit, T.L., Vaewhongs, A.A., Lommel, S.A., 1998. RNA-mediated transactivation of transcription from a viral RNA. *Science* 281, 829–832.
- Solovyeve, A.G., Savenkov, E.I., Grdzeliashvili, V.Z., Kalinina, N.O., Morozov, S.Y., Schiemann, J., Atabekov, J.G., 1999. Movement of hordeivirus hybrids with exchanges in the triple gene block. *Virology* 253, 278–287.
- Stawicki, S.S., Kao, C.C., 1999. Spatial perturbations within an RNA promoter specifically recognized by a viral RNA-dependent RNA Polymerase (RdRp) reveal that RdRp can adjust its promoter binding sites. *J. Virol.* 73, 198–204.
- Van Der Kuyl, A.C., Langereis, K., Houwing, C.J., Jaspars, E.M.J., Bol, J.F., 1990. *Cis*-acting elements involved in replication of alfalfa mosaic virus RNA in vitro. *Virology* 176, 346–354.
- Van Der Vossen, E.A.G., Notenboom, T., Bol, J.F., 1995. Characterization of sequences controlling the synthesis of alfalfa mosaic virus subgenomic RNA in vitro. *Virology* 212, 663–672.
- van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B., 2000. Virus Taxonomy: The taxonomy and nomenclature of viruses. The Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 899–922 and 969–989.
- Verchot, J., Angell, S.M., Baulcombe, D.C., 1998. In vivo translation of the triple gene block of potato virus X requires two subgenomic mRNAs. *J. Virol.* 72, 8316–8320.

- Wang, J., Carpenter, C.D., Simon, A.E., 1999. Minimal sequence and structural requirements of a subgenomic RNA promoter for turnip crinkle virus. *Virology* 253, 327–336.
- Wang, J., Simon, A.E., 1997. Analysis of the two subgenomic RNA promoters for turnip crinkle virus in vivo and in vitro. *Virology* 232, 174–186.
- Watanabe, Y., Ohno, T., Okada, Y., 1982. Virus multiplication in tobacco protoplasts inoculated with tobacco mosaic virus RNA encapsulated in large unilamellar vesicular liposomes. *Virology* 120, 478–480.
- White, K.A., 2002. The premature termination model: a possible third mechanism for subgenomic mRNA transcription in (+)-strand RNA viruses. *Virology* 304, 147–154.
- Zhang, G., Slowinski, V., White, K.A., 1999. Subgenomic mRNA regulation by a distal RNA element in a (+)-strand RNA virus. *RNA* 5, 550–561.
- Zhong, W., Ruekert, R.R., 1993. Flock house virus: down-regulation of subgenomic RNA3 synthesis does not involve coat protein and is targeted to synthesis of its positive strand. *J. Virol.* 67, 2716–2722.
- Zhou, H., Jackson, A.O., 1996a. Analysis of *cis*-acting elements required for replication of barley stripe mosaic virus RNAs. *Virology* 219, 150–160.
- Zhou, H., Jackson, A.O., 1996b. Expression of the barley stripe mosaic virus RNA β “triple gene block.” *Virology* 216, 367–379.